A RAPID DIRECT ASSAY FOR UROPORPHYRINOGEN III COSYNTHETASE

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Received 8 May 1980

1. Introduction

Uroporphyrinogen III (3) the precursor of heme, chlorophyll and vitamin B_{12} is formed from porphobilinogen by the action of two enzymes, porphobilinogen deaminase and uroporphyrinogen III cosynthetase [4]. In the absence of cosynthetase only the uroporphyrinogen I isomer (4) is the formed. Until recently little was known about the individual role played by the two enzymes which were generally thought to function in a complex without the liberation of intermediates between porphobilinogen and uroporphyrinogen III. Consequently uroporphyrinogen III cosynthetase has traditionally been assayed indirectly by the estimation of the isomer ratio between uroporphyrinogen I and III formed, a complex procedure which has greatly hampered the purification and study of the enzyme. Originally uroporphyrin I and III methyl esters were separated by paper chromatography or cellulose thin-layer chromatography (TLC) [5] with unreliable results because of the physical interaction between the two isomers [6]. A more reliable but time consuming separation was accomplished by decarboxylating the uroporphyrins to coproporphyrins which could then be separated and quantified as methyl esters by TLC [7] or high pressure liquid chromatography (HPLC) [8]. The direct separation method of uroporphyrin I and III dimethyl esters [3,9] was a recent important advance which has made the practical routine analysis of cosynthetase more feasible. This latter method has been used by us in the purification and examination of uroporphyrinogen III cosynthetase from *Rhodopseudomonas spheroides* [10]. Although this procedure is a major improvement in both speed and accuracy, it still involves esterification, extraction, TLC and HPLC, leaving a requirement for a rapid assay method for analysis, especially important when large numbers of samples are involved.

In [1] we showed that the role of porphobilinogen deaminase is to form the short lived enzyme free intermediate preuroporphyrinogen (2) ($T_{1/2}$ = 4 min at 37°C, pH 8.2) which acts as the natural substrate for cosynthetase [2] being converted in high yield into uroporphyrinogen III. In the absence of cosynthetase, preuroporphyrinogen spontaneously decomposes into uroporphyrinogen I (4) in a non-enzymic reaction. This finding, namely that the two enzymes act separately and sequentially has enabled the design of a new direct and rapid assay method for uroporphyrinogen III cosynthetase in which preuroporphyrinogen is generated by porphobilinogen deaminase and is converted into uroporphyrinogen III by the cosynthetase. The spontaneous transformation of preuroporphyrinogen into uroporphyrinogen I is limited by the use of short incubation times such that the uroporphyrinogen formed from preuroporphyrinogen is due to the action of the cosynthetase rather than the non-enzymic conversion.

2. Materials and methods

Trisma base was purchased from Sigma Chemical Co. Chromatographic requisites were obtained from Whatman Co. and Bio-Rad Labs. All chemicals were of reagent grade and purchased from Fisher Scientific Ltd. Gilson autopipettes were used.

Porphobilinogen deaminase free of uroporphyrinogen III cosynthetase was purified from *Rodopseudomonas spheroides* according to [11]. The enzyme was stored in 0.1 M Tris–HCl (pH 8.2) (~10 units/ml). One unit of deaminase catalyses the consumption of 1 μmol porphobilinogen/h.

Uroporphyrinogen III cosynthetase was also isolated from *Rhodopseudomonas spheroides* [10]. One unit of cosynthetase catalyses the formation of 1 μmol uroporphyrinogen III/h under the conditions below.

Porphobilinogen lactam was synthesised according to [12]. The lactam was hydrolysed in 2 M KOH and adjusted to pH 5.5 with acetic acid to precipitate the porphobilinogen. The porphobilinogen was washed rapidly with cold water, freeze dried and recrystallised twice to give colourless prisms of porphobilinogen (as the monohydrate).

Uroporphyrinogens were oxidised to uroporphyrins by the addition of a freshly prepared solution of benzoquinone (1 mg/ml in methanol). Excess benzoquinone was reduced by the addition of a saturated solution of sodium bisulfite and the uroporphyrins were measured as follows.

Uroporphyrins were determined spectroscopically at the Soret maximum in a double beam instrument λ_{max} = 399 nm, ϵ_{399} = 224 000 $M^{-1} \cdot cm^{-1}$ or fluorimetrically λ_{ex} = 400 nm; λ_{em} = 615 nm [13].

Samples of uroporphyrin were esterified using BF_3:MeOH and purified by TLC as in [3]. After purification the ratio between isomers I and III were analysed by HPLC [3].

2.1. *Rapid assay for uroporphyrinogen III cosynthetase*

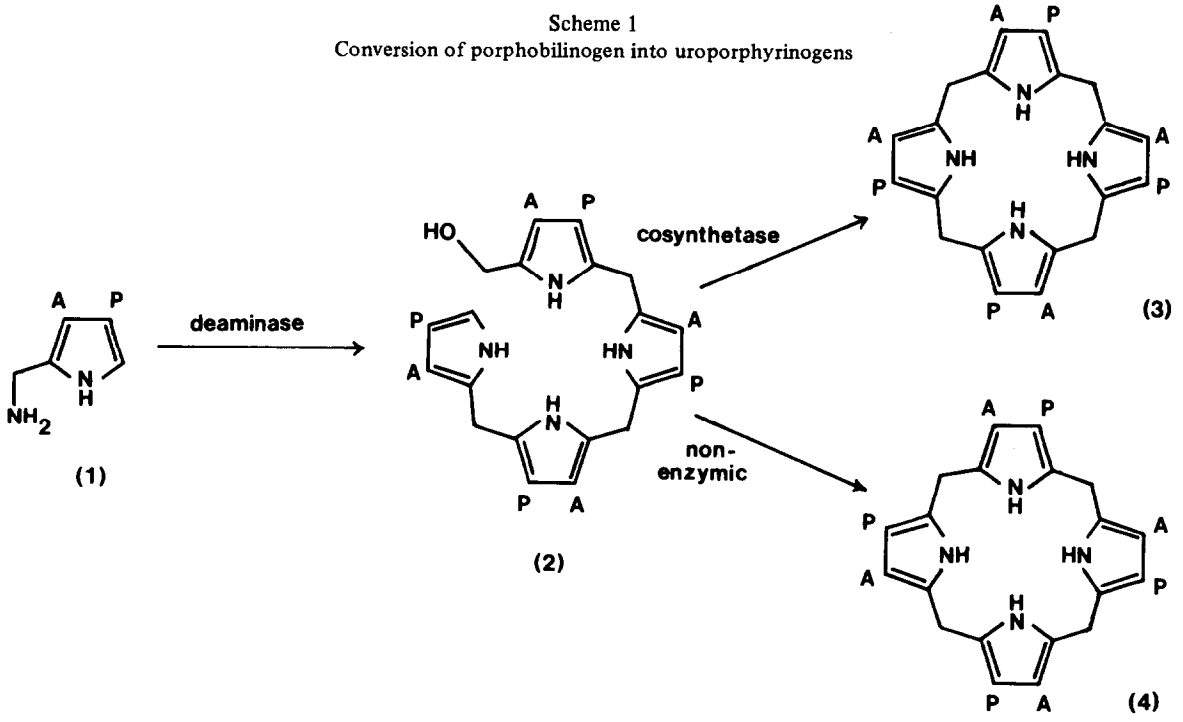
Assays are done in 10 × 100 mm tubes containing 0.1 unit porphobilinogen deaminase, ⩽0.02 units cosynthetase and 10 μmol Tris–HCl (pH 8.2) in 100 μl total vol. After equilibration at 37°C for 1 min the reaction is started by the addition of 10 μl porphobilinogen (2.2 nmol; 0.5 mg/ml in Tris–HCl (pH 8.2)). The incubation is terminated after exactly 2 min by freezing the samples in tubes in a dry ice/acetone slurry. Benzoquinone (50 μl) is added to the frozen solutions which are kept at 0°C for 30 min in the dark. Excess benzoquinone is reduced by the addition of sodium bisulfite (50 μl). The uroporphyrins are diluted by the addition of 2.5 ml Tris–HCl, (pH 8.2) and the uroporphyrins are measured at the Soret maximum. Alternatively the uroporphyrins may be diluted further with Tris–HCl as required and the fluorescence measured as above. Blanks contained no cosynthetase and represent the small amount of uroporphyrinogen I formed from preuroporphyrinogen in the 2 min incubation. Before measurements are carried out either spectrophotometrically or fluorimetrically, a standard solution of uroporphyrin should be employed to calibrate the instruments.

3. Results and discussion

The finding that preuroporphyrinogen (2) the final product of porphobilinogen deaminase is the substrate for uroporphyrinogen III cosynthetase [1,2] has enabled the development of a direct assay system for the cosynthetase. The assay incorporates both porphobilinogen deaminase and uroporphyrinogen III cosynthetase in a coupled system and takes advantage of the fact that the cosynthetase catalyses the formation of uroporphyrinogen III faster than the preuroporphyrinogen is transformed chemically into uroporphyrinogen I (scheme 1). Hence the amount of uroporphyrinogen formed in the presence of both enzymes is greater than the uroporphyrinogen formed in an incubation with deaminase alone. The spontaneous chemical conversion of preuroporphyrinogen into uroporphyrinogen I ($T_{1/2}$ 4 min at 37°C, pH 8.2) is further limited by the use of short incubation times and by rapidly freezing the samples to terminate the reaction. The uroporphyrinogens formed are then oxidised to porphyrins with benzoquinone, conditions under which any remaining preuroporphyrinogen is degraded into non-porphyrin products which exhibit very low absorption (or fluorescence) compared to porphyrins. The amount of uroporphyrinogen III formed is calculated by subtracting the absorbance (or fluorescence) of a blank (deaminase alone) from the values obtained in incubations containing deaminase and cosynthetase (see below).

The uroporphyrinogen III formed as a function of increasing quantities of cosynthetase is shown in fig.1. The relationship is linear up to ~0.02 units of cosynthetase, corresponding to ~5 nmol uroporphyrinogen III (A_{399} 0.448). At higher concentrations of cosynthetase there is a deviation from linearity since the deaminase is unable to maintain the preuroporphyrinogen at a sufficiently high steady state concentration. For accurate determinations of cosynthetase only the values falling on the linear portion of the curve fig.2 should be used although in practice those points on the curved portion (⩽5 nmol) are quite satisfactory for exploratory assays or when only an approximation to units is required. In all cases the blank (no cosynthetase) is subtracted from the values. Typically ~2 nmol uroporphyrinogen I is formed, repre-

Scheme 1
Conversion of porphobilinogen into uroporphyrinogens



senting the maximum amount of preuroporphyrinogen transformed chemically into uroporphyrinogen I. In incubations with cosynthetase the uroporphyrinogen I formed is proportionally less but this only becomes significant at high concentrations of cosynthetase which are outside the assay range.

Parallel determinations in which HPLC [3] was used for determining the ratio between uroporphyrinogen I and III isomers showed excellent agreement with the direct assay determinations particularly when the cosynthetase concentration was low (not shown).

Equally important as the development of the direct assay for cosynthetase is the additional information provided by the above results concerning the mechanism of uroporphyrinogen III formation from porphobilinogen. Until the discovery of preuroporphyrinogen [1,2] the deaminase and cosynthetase were considered to act in a complex, the overall

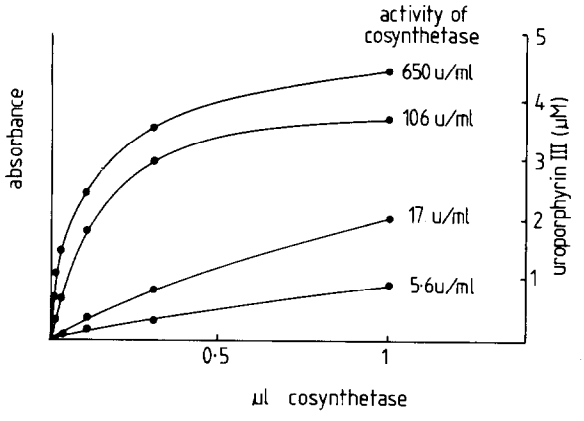


Fig.1. Effect of increasing quantities of uroporphyrinogen III cosynthetase on formation of uroporphyrinogen III. See section 2 for details.

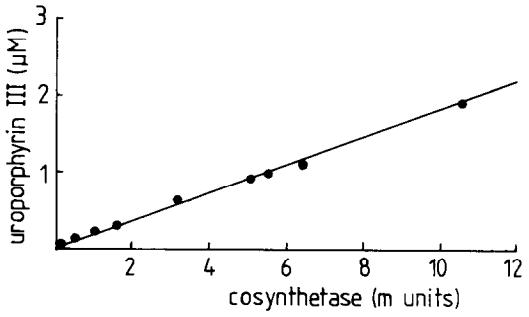


Fig.2. Linear relationship between the formation of uroporphyrinogen III formed and units of uroporphyrinogen III cosynthetase. See section 2 for details.

conversion occuring without the involvement of any free intermediates [15]. The asymptotic relationship between uroporphyrinogen III formed and uroporphyrinogen III cosynthetase utilized (fig.1) is typical of a coupled reaction with enzymes acting in sequence (scheme 1), thus providing additional evidence for the hypothesis originally deduced from [2], namely (1)→(2)→(3).

The utility of the assay is highlighted by the fact that it is possible to determine 50 samples of cosynthetase in <1 h, a dramatic improvement over earlier methods. The assay as described in addition to the investigation of kinetic aspects may be used for the determination of cosynthetase in crude homogenates or lysates and may be employed clinically for the estimation of cosynthetase in blood or in samples from biopsy. In these cases the fluorimetric method is more sensitive. Some care must be exercised when investigating samples in which endogenous uroporphyrinogen III decarboxylase or porphobilinogen deaminase are excessively high.

Acknowledgements

We thank for National Institute of Health (grant AM-205 28) and the Robert A. Welch Foundation for the support of this work, the Consejo Nacional de Investigaciones Cientificas y Tecnicas de La Republica Argentina for a fellowship to G. B. and the American Philosophical Society to P. M. J. The technical assistance of R. C. Lewis and L. Pryde is appreciated.

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